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Germline mutations in the BRCA2 gene lead to an increased lifetime risk for breast and ovarian cancer, and pancreatic, prostate, and male breast cancers. Although the BRCA2 protein has been reported to play a role in DNA repair, the normal cellular function of the BRCA2 gene is still mostly unknown. In this report, we demonstrate that cellular levels of BRCA2 protein are diminished following UV irradiation, suggesting that regulation of BRCA2 during DNA repair is complex. In work based on a previous collaborative observation, we also explore a potential interaction between BRCA2 and Braf35 proteins and provide evidence that this interaction does not occur in cells. Additionally, we show that among the human homologs of the RecA family of DNA repair proteins, BRCA2 binds exclusively to Rad51. Regulation of BRCA2 expression by p53 was assessed and BRCA2 levels were found to be unaffected by p53. We also report the construction of a series of epitope-tagged BRCA2 constructs that will be used to identify potential BRCA2-interacting proteins. These efforts will help provide a more complete picture of the normal cellular role of the BRCA2 gene.

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Alan Schaffer 3-27-01

PI - Signature Date

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Introduction

The breast cancer susceptibility genes, BRCA1 and BRCA2, have been implicated in early-onset familial breast and ovarian cancers (1, 2). Inherited mutations in BRCA1 and BRCA2 result in an increased lifetime risk for breast cancer (3) and are responsible for the vast majority of hereditary breast cancers [reviewed in (4)]. Germline mutations in BRCA2 are also associated with an increased risk of male breast cancer (5-8), prostate cancer, and pancreatic cancer [(4) and references therein].

BRCA2, like BRCA1, is a classical tumor suppressor gene, requiring inactivation of both alleles to progress toward tumorigenesis (9). Thus, affected individuals inherit one mutant copy of the allele. Loss of the wild-type allele, which can be detected in breast tumor tissue, leads to tumor formation (10). Additionally, loss of heterozygosity (LOH) at the BRCA2 locus (on chromosome 13q12-13) is frequently detected in sporadic cases of breast and ovarian cancer (reviewed in (11)). However, somatic mutations in BRCA2 and BRCA1 are very rare in sporadic breast cancers (1, 12, 13).

The BRCA2 gene is extremely large with a complex genomic structure consisting of one noncoding exon and 26 coding exons, encoding a protein of 3418 amino acids (6). BRCA2 is expressed (although at low levels) in many tissue types, with the greatest expression in the testis, thymus, spleen, and ovaries (6, 14). BRCA2 expression is cell cycle-regulated, with the highest levels detected at the G1/S boundary (15-17). Additionally, BRCA2 expression is coordinately up-regulated with BRCA1 in proliferating and differentiating cellular compartments (14) and in mammary cells (15), indicating that the two breast cancer susceptibility genes might function in common or overlapping cellular pathways. Furthermore, the BRCA2 gene is regulated during mammary development, with induction seen during puberty and pregnancy, but not during lactation (14).

Targeted disruption of the BRCA2 gene in mice leads to embryonic lethality, due to an apparent defect in cellular proliferation (18-20). However, mice homozygous for an allele which leads to truncation of BRCA2 in exon 11 can survive at some frequency, although they are born with deformities and eventually die at an early age from thymic lymphomas (21, 22). Interestingly, this region of BRCA2 (exon 11) contains eight repetitive units termed BRC repeats (23). Six of these BRC repeats have been shown to be capable of binding to the RAD51 protein, which has been implicated in DNA double-stand break repair (24). Along these lines, BRCA2 knockout embryos are hypersensitive to gamma-irradiation (although the C-terminal region of mouse BRCA2 was reported to bind to RAD51) (18). Additionally, mouse embryo fibroblasts (MEFs) from homozygous null BRCA2 mice accumulate gross chromosomal rearrangements (25, 26). This cumulative data suggests a role for BRCA2 in DNA repair or maintenance.

Attempts to understand the function and biochemical properties of the endogenous BRCA2 protein have been hampered by its large size and a lack of suitable immunological reagents for its detection. Toward this end, our laboratory generated and characterized two polyclonal and three monoclonal antibodies (mAbs) against BRCA2. The two polyclonal antibodies were generated against peptides at the N-terminus (N-19) and C-terminus (C-15) of BRCA2. The mAbs were generated against GST-fusion proteins corresponding to regions in the N-terminus, middle, and C-terminus of BRCA2. Using these antibodies, we determined that the BRCA2 gene product is a 460-kDa nuclear phosphoprotein that associates *in vivo* with a significant portion of the endogenous pool of RAD51. Given the role of RAD51 in repair of DNA damage, we also assayed whether BRCA2 associates with p53, which is also important for the cellular response to DNA damage. Indeed, a physical and functional relationship between BRCA2 and p53 was demonstrated. These results have been previously reported in our previous annual fellowship report [and in (27)].

In this report, we demonstrate that cellular levels of BRCA2 protein are diminished following UV irradiation. In work based on a previous collaborative observation, we also explore a potential interaction between BRCA2 and Braf35 proteins and provide evidence that this interaction does not occur in cells. Additionally, we show that among the human homologs of the RecA family

of DNA repair proteins, BRCA2 binds exclusively to Rad51. Regulation of BRCA2 expression by p53 was assessed and BRCA2 levels were found to be unaffected by p53. We also report the construction of a series of epitope-tagged BRCA2 constructs that will be used to identify potential BRCA2-interacting proteins. These efforts will help provide a more complete picture of the normal cellular role of the BRCA2 gene.

Body of the Report

Experimental Procedures

Cell culture and transfections. All cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). 293T cells plated in 35-mm culture dishes were transfected for 5 hrs with 1 μg of DNA, 6 μl of Lipofectamine Plus reagent, and 4 μl of Lipofectamine (Life Technologies, Gaithersburg, MD) in 1 ml of Opti-MEM reduced serum medium (Life Technologies, Gaithersburg, MD). For most experiments, transfected cells were then replated into either 100-mm culture dishes (for immunoprecipitations) or multiple 35-mm dishes (for UV experiments) and allowed to grow for 3 days prior to assays.

UV irradiation. Cells were UV-irradiated by aspirating medium and exposing to a 254 - nm germicidal lamp inside a tissue culture hood. Some cells were incubated with the proteasome inhibitor, MG132 (Calbiochem), which was dissolved in DMSO at a concentration of 20 mM and diluted 1:400 with culture medium to a final concentration of 50 mM.

Plasmids. The GFP-BRCA2 plasmid has been previously described (27). Constructs directing expression of HA-tagged proteins were all created by PCR amplification of appropriate cDNAs with forward primers containing an in-frame *Bam*HI site and reverse primers containing an *Xho*I site. PCR products were digested with the indicated restriction enzymes and placed into a similarly digested pcDNA3-HA vector, which contains the HA epitope upstream of a multiple cloning region, allowing for N-terminally tagged products. Flag-tagged BRCA2 fragments (1-936; exon 15 to end) were created by PCR amplification of the GFP-BRCA2 plasmid with forward primers coding for an N-terminal Flag epitope (MDYKDDDDK) and reverse primers containing a stop codon. PCR products were directionally cloned into *Kpn*I and *Xho*I sites in the pcDNA3 vector (Invitrogen).

Immunoprecipitations. All steps were done at 4°C. Cells grown in 100-mm culture dishes were lysed in 0.5 ml of lysis buffer (50 mM Hepes, pH 7.6; 250 mM NaCl; 0.1% Nonidet P-40 (NP-40); 5 mM EDTA; 1 mM phenylmethylsulfonyl fluoride (PMSF); 1mM sodium orthovanadate; 1 μg/ml each of aprotinin, bestatin and leupeptin) for 30 min at 4°C. Lysates were clarified by microfuge for 15 min at 4°C. The total protein content of the lysates was determined by the BCA assay (Pierce). 2 mg of the clarified lysates were incubated for 1.5 hours with either 5 μg of purified monoclonal or polyclonal antibody or 100 μl of mAb supernatant. Where mAbs were used, after 1 hour of incubation, 2 μg of rabbit anti-mouse IgG was added to the immunoprecipitation. All immune complexes were collected by incubating for 1.5 hours with 100 μl of a 15% slurry of protein A-Sepharose (Pharmacia, Uppsala, Sweden). Immune complexes were then washed four times with lysis buffer, heated to 70°C for 7 min in SDS loading buffer, and separated by SDS-PAGE. Proteins were then visualized by immunoblotting. For immunoprecipitations that were analyzed in two parallel gels, double amounts of all materials were used, and the resulting immune complexes were divided evenly among the two gels.

Immunoblotting. After electrophoresis, proteins were transferred onto a poly(vinylidene difluoride) (PVDF) membrane (Millipore). To allow immunoblotting with more than one antibody, some membranes were cut horizontally (according to the molecular mass markers) and the resulting pieces of membrane (containing the appropriate molecular size range for the protein to be detected) were immunoblotted separately. All membranes were blocked for 1 hour at room temperature in 8% nonfat milk in TBS containing 0.5% Tween 20 (TBS/Tween). Membranes were then incubated for 1 hr at room temperature with a primary antibody diluted in 2% milk in TBS/Tween. After removing the primary antibody, membranes were washed 3 times (10 min each) with TBS/Tween, incubated for 1 hr with a secondary antibody in 1% milk/TBS/Tween and washed 3 times. ECL or ECLPlus (Amersham) was used for signal detection.

Antibodies. For immunoprecipitations, the following antibodies were used: Affinity purified BRCA2 mAb B2 (27) was generated against a GST-BRCA2 fusion protein containing amino acids 1651 to 1820. Braf35 mAb 11C12 supernatant was generated from mice immunized with a Braf35-GST fusion protein. Anti-Flag M2 mAb (Sigma), Rad51 polyclonal antibody (Oncogene Research) and p21 mAb (Oncogene Research) were obtained from their manufacturer. Anti-HA (12CA5) and anti-p53 mAbs (1801) were produced at Mount Sinai Medical Center. For immunoblotting, the following antibodies were used: Affinity purified BRCA2 polyclonal antibody C15, directed against the 15 amino acids at the extreme C-terminal of BRCA2 (27), was used at a 1:500 dilution. Braf35 mAb 1B5 supernatant, generated from mice immunized with a Braf35-GST fusion protein, was used at a 1:5 dilution.

Results

UV irradiation causes a decrease in BRCA2 protein levels. The BRCA2 protein is likely to be involved in DNA repair or maintenance. In order to define the role of BRCA2 in this cellular process, we examined the expression of BRCA2 following DNA damage. Previous studies had shown that BRCA2 mRNA is down-regulated following exposure to UV irradiation (28, 29). In order to test whether the BRCA2 protein is affected similarly, and to define the kinetics of BRCA2 regulation following UV damage, we exposed 293T and MCF7 cells to UV (Figure 1).

In 293T cells, UV caused a rapid decrease in levels of BRCA2 protein (Figure 1A). Within 2 hours following UV, BRCA2 levels were almost undetectable. Since this effect may occur through down-regulation of the BRCA2 promoter, 293T cells were transiently transfected with a GFP-BRCA2 expression construct, which utilizes a heterologous promoter. Diminution of the GFP-BRCA2 product occurred with similar kinetics as the endogenous BRCA2 protein (Figure 1A), suggesting that this effect does not occur at the level of transcription initiation (*i.e.* a promoter effect). To examine whether the observed decrease occurred through proteasomal degradation of BRCA2 protein, the experiment was repeated in the presence of the proteasome inhibitor, MG132 (Figure 1A, +MG132). However, the reduction in BRCA2 protein was only partially blocked by MG132. Thus, proteasomal degradation does not appear to play a major role in regulation of BRCA2 in response to UV-mediated DNA damage in 293T cells.

Since 293T cells contain viral proteins which inactivate p53, which may be important for the cellular response to UV, we also performed experiments using MCF7 breast cancer cells. MCF7 cells also displayed a UV-dependent reduction in BRCA2 protein levels, with slightly different kinetics (Figure 1B). At 2 hours post-UV, BRCA2 levels were equivalent to those of untreated cells. BRCA2 protein levels were significantly diminished at 4 hours after UV treatment. As previously, MG132 had little effect on the UV-mediated reduction of BRCA2 protein levels in MCF7 cells. As a control for the UV response, we also assayed for p53 protein. A UV-dependent increase in p53 levels was observed, as expected. Surprisingly, levels of the p53-inducible protein, p21, were seen to diminish following UV treatment. However, unlike BRCA2, p21 levels were restored by MG132, providing a control for the proper function of the proteasome inhibitor.

BRCA2 and Braf35 do not specifically interact. In our previous annual fellowship report, Dr. Yingcai Wang from our laboratory reported that, in collaboration with Dr. Ramin Shiekhattar (Wistar Institute), he had identified a novel BRCA2-associated protein, Braf35 (BRCA2 Associated Factor 35). In order to further investigate a potential interaction between BRCA2 and Braf35, we performed co-immunoprecipitation experiments (Figure 2).

Our laboratory generated monoclonal antibodies to Braf35 (as described in Experimental Procedures) to use in these analyses. 293T cells were either untransfected or transfected with HA-Braf35 or HA-Rad51 expression constructs. Lysates from these cells were immunoprecipitated with antibodies to BRCA2, Braf35, Rad51, or Flag epitope (as a negative control). In untransfected 293T cells (Figure 2, lanes marked 293T), the endogenous Braf35 protein was not detected in BRCA2 immune complexes (Figure 2, top panel). Conversely, BRCA2 was not observed to co-immunoprecipitate with endogenous Braf35 (Figure 2, top and bottom panels). As a positive

control for the immunoprecipitation procedure, Rad51, which has been shown to bind to the BRCA2 protein (24, 27), was also immunoprecipitated. BRCA2 was detected in Rad51 immune complexes (Figure 2, top and bottom panels), as expected, indicating that BRCA2 interacting proteins can be detected by our immunoprecipitation protocol.

When co-immunoprecipitations were performed on lysates from cells over-expressing an HA-Braf35 product (Figure 2, lanes marked 293T HA-Braf35), a BRCA2 immune complex did contain a low level of HA-Braf35 (Figure 2, top panel). However, the control Flag immunoprecipitation also contained a similar amount of HA-Braf35 (Figure 2, top panel), indicating that HA-Braf35 was bound nonspecifically in the immunoprecipitation reaction. Furthermore, although a large amount of Braf35 (endogenous and HA-Braf35) was immunoprecipitated with Braf35 antibody from this lysate, no BRCA2 was detected in this immune complex (Figure 2, top panel). In contrast, HA-Rad51 specifically co-immunoprecipitated with BRCA2 in lysates from cells expressing HA-Rad51 (Figure 2, lanes marked 293T HA-Braf35) and conversely, an increased quantity of BRCA2 protein was detected in Rad51 immune complexes. Thus, a specific BRCA2-Braf35 interaction was not seen in these experiments.

In order to rule out the possibility that the BRCA2 and Braf35 antibodies utilized in the previous co-immunoprecipitations were not capable of binding BRCA2-Braf35 complexes due to steric hindrance, experiments were performed in which proteins were immunoprecipitated via an HA-epitope tag (Figure 3). 293T cells were transfected with HA-TCF (as a negative control), HA-Braf35, and HA-Rad51 (as a positive control) expression constructs and the resulting lysates were immunoprecipitated using HA mAb 12CA5. However, no BRCA2 was detected in HA-Braf immune complexes (Figure 3), whereas BRCA2 did specifically co-immunoprecipitate with HA-Rad51, as expected. These results indicate that there is no specific interaction between BRCA2 and Braf35 proteins.

Analysis of complexes between BRCA2 and DNA repair proteins. Since BRCA2 is likely to be involved in DNA repair and is capable of forming complexes with Rad51, we investigated whether BRCA2 can interact with other members of the family of human DNA repair proteins with homology to the *E. Coli* RecA product (Figure 4). 293T cells were transfected with HA-XRCC2, HA-XRCC3, HA-DMC1, HA-Braf35 (as a negative control), and HA-Rad51 and the resulting cell lysates were immunoprecipitated with HA antibody. BRCA2 was detected solely in HA-Rad51 complexes (Figure 4). In this experiment XRCC2 was not expressed, however no BRCA2-XRCC2 interaction was detected in separate experiments (data not shown). Interestingly, endogenous Rad51 was seen to co-immunoprecipitate with HA-Rad51 (Figure 4). This suggests either that Rad51 can exist in cells as a dimer or that multiple Rad51 proteins can simultaneously interact with BRCA2. Since the current data does not provide evidence supporting either of these possibilities, this observation will be investigated further.

p53 does not down-regulate BRCA2 protein levels. Our laboratory has previously determined that BRCA2 protein functionally interacts with p53 (27). Furthermore, in collaboration with the laboratory of Sam Lee (Harvard Institutes of Medicine), our laboratory has seen that p53 can modulate the expression of BRCA1 (30). To explore a potential role of p53 in regulation of BRCA2 expression, we utilized EJ-p53 cells, in which p53 production is under the control of a tetracycline-repressible promoter construct (31). Thus, p53 is induced upon removal of tetracycline from the culture medium.

EJ-p53 cells were grown for one and two days in the presence and absence of tetracycline (Figure 5). Removal of tetracycline led to accumulation of p53 protein and of the p53-inducible protein, p21. However, levels of BRCA2 were unaffected by the expression of p53 in these cells (Figure 5).

Expression of Flag-tagged BRCA2 products. Although the BRCA2 protein is large and is likely to interact with numerous other proteins, few have been shown to specifically bind to BRCA2. Identification of novel BRCA2-interacting proteins has been difficult due to the large size of BRCA2 and a lack of suitable immunological reagents. Researchers in our lab have been successful at immunoprecipitation of Flag-tagged proteins (32) using commercially available beads containing covalently linked anti-Flag antibodies (Sigma). Toward this end, we have begun to create Flag-tagged BRCA2 expression constructs (Figure 6). In order to avoid the problems

inherent with large proteins, we will construct a series of plasmids directing expression of smaller, overlapping segments of the BRCA2 coding region, covering the entire protein. In this manner, proteins which complex with BRCA2 will not only be identified, but the interacting domain of BRCA2 will also be mapped. This study of BRCA2-interacting proteins will provide insight into the normal cellular function of BRCA2 and a better understanding of how loss of this function leads to tumorigenesis.

Discussion

Our data indicates that levels of the BRCA2 protein are decreased following exposure to UV irradiation. Surprisingly, an exogenously expressed GFP-BRCA2 construct underwent UV-mediated down-regulation with similar kinetics. Since this construct does not contain the endogenous BRCA2 promoter, this suggests that BRCA2 expression is not down-regulated at the level of transcription initiation. This prompted us to explore the possibility that this effect occurs through degradation of BRCA2 protein by the proteasome. However, proteasomal degradation only partially accounted for the diminished levels of BRCA2 protein. Moreover, it has been clearly demonstrated that UV causes a decrease in BRCA2 mRNA (28, 29). Since GFP-BRCA2 and endogenous BRCA2 proteins were diminished similarly after UV exposure, the cumulative data suggests that BRCA2 mRNA stability is reduced following UV and that the region of BRCA2 mRNA controlling this effect is within the BRCA2 protein coding sequence (the region in common between endogenous BRCA2 and GFP-BRCA2). Alternatively, the large size of the RNA transcript may play a role in mediating the UV effect.

The observed decrease in the levels of BRCA2 protein in response to UV is in opposition to an anticipated role of BRCA2 in repair of UV-mediated DNA damage. However, BRCA2 may be necessary in the initial response to DNA damage (before 2 hours), and the reduction of BRCA2 levels may occur at a time when BRCA2 protein is either no longer needed or its presence impedes some downstream DNA repair event. Further studies are necessary to clarify the mechanism and functional significance of the observed down-regulation of BRCA2 following UV exposure.

Expression of p53 was seen to have no effect on the levels of BRCA2 in the cell. This is unexpected, given the documented interplay between p53 and BRCA2 in transcriptional regulation (27) as well as in mouse development (19-21). The experiments reported here were performed with a p53-negative cell line (mutationally inactivated) in which p53 expression was restored by a tetracycline-repressible construct (31). However, leaky expression of tetracycline-regulated proteins can occur. Thus, when tetracycline was included in the culture medium, some residual level of p53 (although not fully detectable by immunoblotting) may have been produced which could have affected BRCA2 levels. Removal of tetracycline and full induction of p53 would then have no effect on BRCA2 levels in this case, as residual p53 may have already reduced BRCA2 levels. Given the importance of p53 in human cancers and the reported relationships between p53 and BRCA2, further experimentation is needed to corroborate the current findings and/or to uncover the true mechanisms by which the p53 and BRCA2 proteins functionally interact.

We have performed a thorough analysis of the potential physical association of BRCA2 and Braf35 proteins. We consistently find no interaction between these two products in the cell. In fact, relatively few proteins have been shown to specifically complex with BRCA2. Interactions between BRCA2 and Rad51 (18, 24, 27, 33), P/CAF (34), and BRCA1 (35) have been reported. However, it is likely that many more BRCA2-interacting proteins will be discovered, given its large size. Toward this end, we have generated Flag-tagged BRCA2 expression constructs and these reagents will be utilized in co-immunoprecipitation experiments to identify new members of the BRCA2 protein complex. Through the study of BRCA2-interacting proteins, a more comprehensive picture of BRCA2 function will emerge, providing a better understanding of the mechanisms by which inactivation of the BRCA2 gene leads to tumor formation.

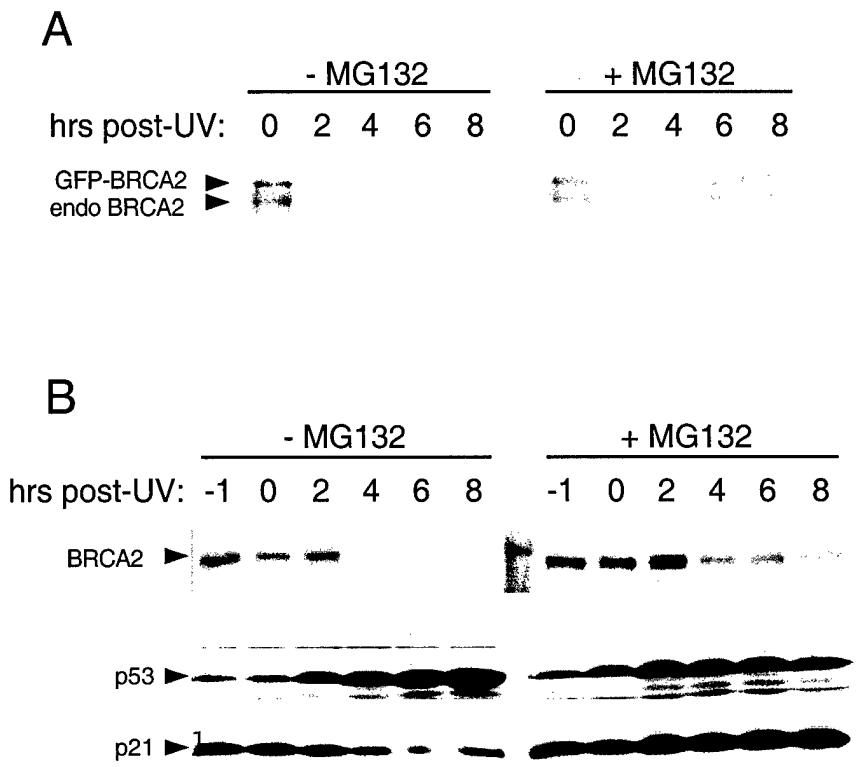

Figure 1. Effect of UV on BRCA2 protein levels. Cells were exposed to UV-C irradiation (as indicated in Experimental Methods) and then incubated in the absence (- MG132) or presence (+ MG132) of proteasome inhibitor. Cells were then collected at the time points indicated above the blot and lysates were prepared. Each lane was normalized for equal protein loading (200 μg). BRCA2 immunoblots were performed using polyclonal rabbit antibody C-15. (A) 293T cells transiently transfected with a GFP-BRCA2 expression plasmid were exposed to 30 J/m^2 of UV. Positions of the GFP-BRCA2 and endogenous (endo) BRCA2 products are indicated to the left of the blot. (B) MCF7 cells were incubated for 1 hour in the absence (- MG132) or presence (+ MG132) of proteasome inhibitor (at time -1 hr). Cells were then exposed to 60 J/m^2 of UV at time 0 hr. Position of BRCA2, p53 and p21 are indicated to the left of the blot.

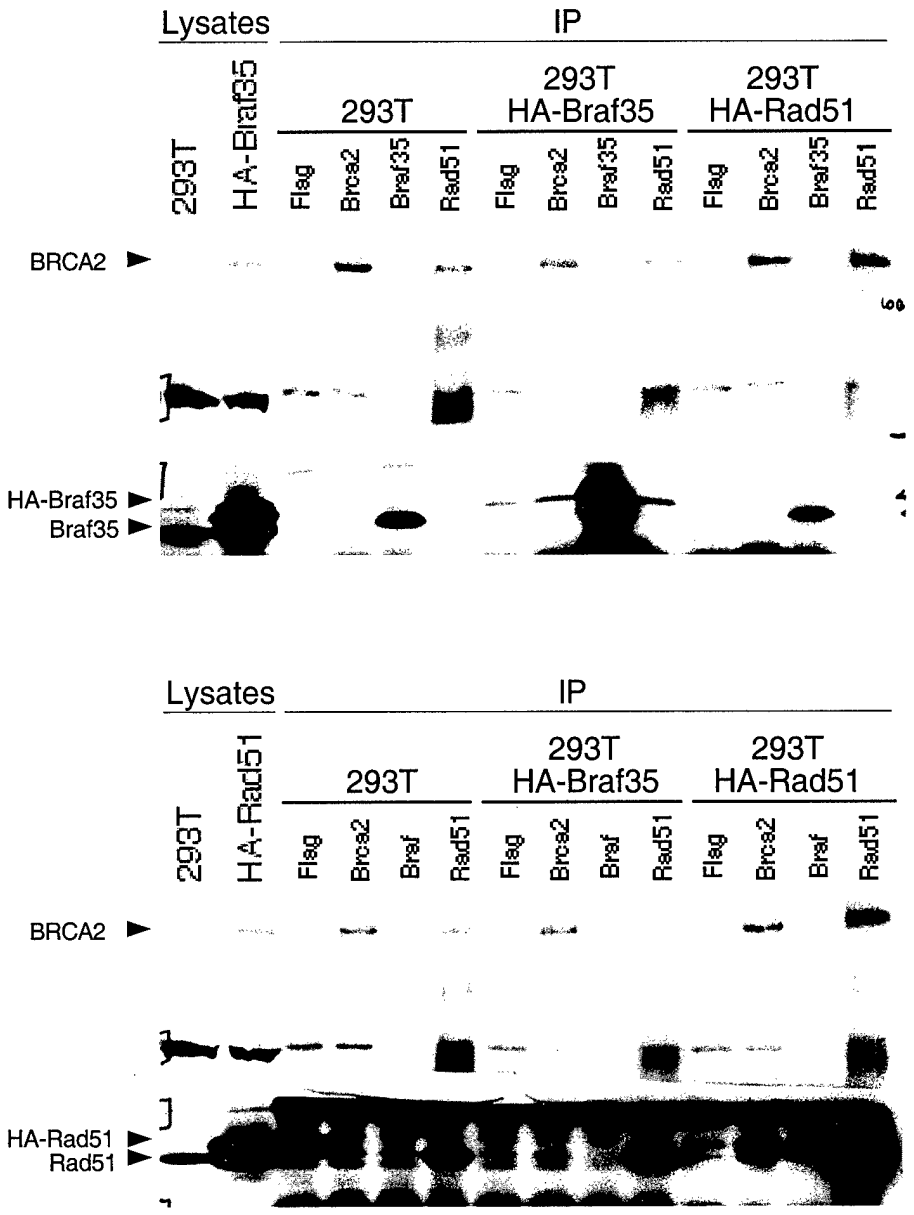

Figure 2. Lack of interaction between BRCA2 and Braf35. Immunoprecipitations (lanes marked IP) were performed (as described in Experimental Procedures) on 293T cells, either untransfected, or transfected with HA-Braf35 or HA-Rad51 constructs (as indicated above each blot) with antibodies to Flag (as a negative control), BRCA2, Braf35, or Rad51 (as indicated at the top of each lane). Immune complexes were divided and electrophoresed on two split 5%/11% polyacrylamide-SDS gels (top and bottom panels). As size controls, lysates (200 μg) from untransfected (top and bottom panels), HA-Braf35 transfected (top panel), or HA-Rad51 transfected (bottom panel) 293T cells were also loaded onto the gel (lanes marked lysates). After transfer, membranes were cut and the top halves were immunoblotted with BRCA2 polyclonal antibody C15 (top and bottom panels). The bottom halves of the membrane were immunoblotted with antibodies to Braf35 (top panel) or Rad51 (bottom panel). Positions of BRCA2, HA-Braf35, endogenous Braf35, HA-Rad51, and endogenous Rad51 are indicated to the left of the blots.

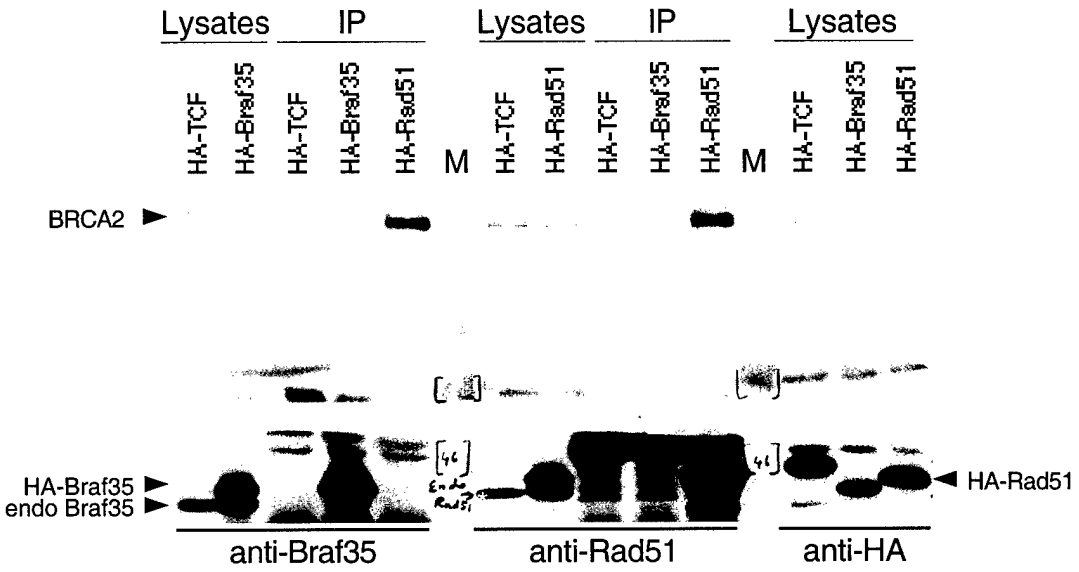

Figure 3. Lack of interaction between BRCA2 and Braf35 in HA immunoprecipitations. 293T cells were transfected with HA-TCF (as a negative control), HA-Braf35 or HA-Rad51 constructs. Lysates from transfected cells (as indicated above each lane) were immunoprecipitated with HA mAb 12CA5. Immune complexes (lanes marked IP) or lysates (75 μg, lanes marked lysates) were electrophoresed on a split 5%/11% polyacrylamide-SDS gel. After transfer, the membrane was cut horizontally. The top half was immunoblotted with BRCA2 polyclonal antibody C15. The bottom half of the membrane was cut vertically along the marker lanes (M) and the resulting pieces were immunoblotted with antibodies to Braf35, Rad51, or HA (as indicated at the bottom of each blot). Positions of BRCA2, HA-Braf35, and endogenous (endo) Braf35 are indicated to the left of the blot. Position of HA-Rad51 is indicated to the right. Endogenous Rad51 is indicated with a white *.

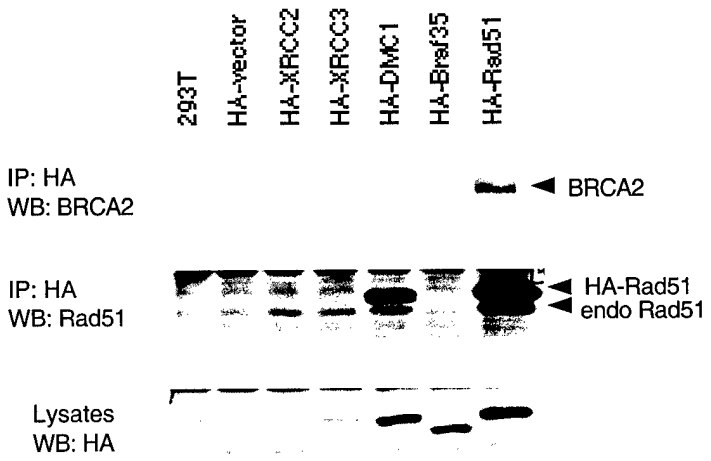

Figure 4. Analysis of BRCA2 interaction with DNA repair proteins. Lysates from 293T cells, either untransfected or transfected with empty HA-vector or HA-XRCC2, HA-XRCC3, HA-DMC1, HA-Braf35, or HA-Rad51 constructs (as indicated above each lane) were immunoprecipitated with HA mAb 12CA5. Immune complexes (panels marked IP) or lysates (75 μg, panel marked lysates) were electrophoresed on a split 5%/10% polyacrylamide-SDS gel. The top half of the membrane was immunoblotted with BRCA2 polyclonal antibody C15 (top panel). The bottom half was immunoblotted with Rad51 antibody (middle panel). Note that DMC1 cross-reacts with Rad51 antibody. Lysates were (75 μg) were separately electrophoresed and immunoblotted with HA antibody (bottom panel). Positions of BRCA2, and HA-Rad51, and endogenous (endo) Rad51 are indicated to the right. Note that although HA-XRCC3 was not abundantly expressed, immunoprecipitation of HA-XRCC3 was equivalent to that of HA-Rad51 (data not shown).

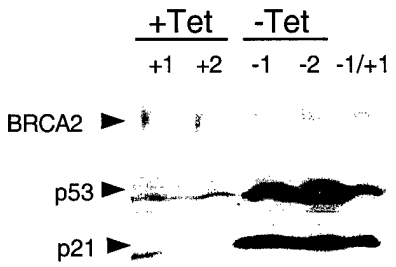

Figure 5. Effect of p53 expression on BRCA2 levels. EJ-p53 cells were plated at a low cell density in the presence of tetracycline (1 μg/ ml final concentration) one day before the start of experiment (day 0). At day 0, cells were washed 3 times with PBS and incubated in media in which tetracycline was either included (+Tet) or excluded (-Tet) for 1 or 2 days (as indicated above lanes). Lane marked -1/+1 was incubated without tetracycline for 1 day and then incubated for 1 day in media containing tetracycline. Each lane was normalized for equal protein loading (200 μg). Lysates were electrophoresed on a split 5%/10% polyacrylamide-SDS gel. After transfer, the membrane was immunoblotted with antibodies to BRCA2, p53, or p21 (as indicated to the left)

Figure 6. Expression of Flag-tagged BRCA2 products. Lysates (200 μg) from 293T cells, either untransfected or transfected with Flag-tagged BRCA2 constructs (as indicated above lanes) were electrophoresed on a 7% SDS-PAGE gel. Immunoblotting was performed with Flag mAb M2. Positions of Flag-tagged BRCA2 products are indicated by arrows to the right of the blot. Positions of molecular mass standards (kDa) are indicated to the left.

Key Research Accomplishments

- Characterized the BRCA2 gene product as a 460 kDa phosphoprotein that forms a complex with Rad51 *in vivo*.
- Uncovered a functional interaction between BRCA2 and p53, namely that BRCA2 inhibits p53 transcriptional activation.
- Showed that Rad51 enhances the BRCA2-mediated inhibition of p53 transactivation.
- Described a decrease in BRCA2 protein levels following UV irradiation.
- Demonstrated that the BRCA2 protein does not interact with Braf35.
- Provided evidence that among human homologs of the RecA family of DNA repair proteins, BRCA2 binds exclusively to Rad51.

Reportable Outcomes

- Characterization of BRCA2 protein and the functional interaction of BRCA2 with p53 and Rad51 have been reported both in a journal (Marmorstein, L.Y., Ouchi, T., and Aaronson, S. A. (1998). The *BRCA2* gene product functionally interacts with p53 and RAD51. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13869-13874) and at a scientific meeting at Cold Spring Harbor Laboratories (Marmorstein, L.Y., Ouchi, T., Bartel, P. and Aaronson, S. A. (1998). The nuclear phosphoprotein BRCA2 inhibits p53 activity synergistically with RAD51).
- Monoclonal and polyclonal antibodies to the BRCA2 protein have been developed.
- Support for training has been provided (at least in part) for the following postdoctoral fellows: Lihua Y. Marmorstein, Yingcai Wang, Alka M. Mahale, and Alan R. Schoenfeld.

Conclusions

In these studies, we have identified the *BRCA2* gene product as a 460 kDa nuclear phosphoprotein that forms a complex with RAD51 in vivo. These findings established that a major fraction of endogenous RAD51 is associated with the endogenous BRCA2 protein, implying that this interaction is functionally significant. Furthermore, we have demonstrated that CAPAN-1 tumor cells, which contain a *BRCA2* mutation producing a truncated product, express a single BRCA2 immunoreactive species of approximately 230 kDa, consistent with its predicted size. Of note, the mutant protein, though expressed at low level, interacted with RAD51 as efficiently as the wild type BRCA2. This mutant would be predicted to retain all eight repetitive BRC motifs which serve as RAD51 binding sites in human BRCA2 (36). These results point to a possible mechanism by which truncated BRCA2 mutants found commonly in tumors (Breast Cancer Information Core) may actually sequester RAD51 in a nonfunctional complex.

We have established that p53 exists *in vivo* in complexes containing BRCA2. Moreover, a functional interaction between BRCA2 and p53 was demonstrated in that BRCA2 inhibits p53 transcriptional activity. These results are consistent with the possibility that BRCA2 may act to limit the length or severity of p53-mediated cell cycle arrest following DNA damage. We did not detect any effect of RAD51 alone on p53 transcriptional activity. However, cooperative down-regulation of p53 transcriptional activity by RAD51 and BRCA2 was observed, providing evidence that RAD51 is coupled to transcription pathways through its interactions with BRCA2 and p53. Thus, BRCA2 appears to serve as a regulator linking both cell cycle control and DNA repair pathways.

In studies performed since the last annual report, the function of BRCA2 gene has been further investigated. BRCA2 expression has been examined under conditions of DNA damage and in the presence or absence of the p53 protein. Biochemical analyses of the BRCA2 protein have been performed through studies of BRCA-interacting proteins. The conclusions of the present studies are as follows:

1) UV irradiation causes a decrease in BRCA2 protein that occurs partly through degradation of BRCA2 protein level, but primarily at the mRNA level. BRCA2 down-regulation probably occurs through destabilization of BRCA2 mRNA.

2) BRCA2 does not interact with the Braf35 protein. Furthermore, among the human homologs of RecA, BRCA2 interacts exclusively with Rad51.

3) p53 expression does not affect the levels of BRCA2 protein in cells.

4) The future study of BRCA2-interacting proteins through the use of Flag-tagged segments of BRCA2 protein will provide a better understanding of the normal cellular function of BRCA2.

The studies supported by this award have provided the first evidence of quantitative physiological interactions between endogenous BRCA2 and RAD51 and the first demonstration of complexes and functional interactions involving BRCA2 and p53. Additionally, these studies have provided the first evidence that BRCA2 is a phosphoprotein and the first production of monoclonal antibodies with which to immunoprecipitate and characterize BRCA2. These antibodies should be valuable to the entire field of BRCA2 research. Moreover, the characterization of the BRCA2 gene product in these studies provides a basis for future investigations on the function of BRCA2.

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Appendices

Copies of an abstract and a journal article resulting from the work funded by this grant are attached:

1. Abstract:
   Marmorstein, L.Y., Ouchi, T., Bartel, P. and Aaronson, S. A. (1998). The nuclear phosphoprotein BRCA2 inhibits p53 activity synergistically with RAD51. Pathways to cancer, 30. (Cold Spring Harbor Laboratory).

2. Journal article:
   Marmorstein, L.Y., Ouchi, T., and Aaronson, S. A. (1998). The *BRCA2* gene product functionally interacts with p53 and RAD51. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13869-13874.

THE NUCLEAR PHOSPHOPROTEIN BRCA2 INHIBITS p53 ACTIVITY SYNERGISTICALLY WITH RAD51

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Germline mutations in the human BRCA2 gene confer susceptibility to early-onset familial breast cancers. Mouse embryonic fibroblasts lacking BRCA2 are defective in proliferation presumably mediated by over-expression of p53 and $p21^{WAF1/CIP1}$. These cells are also hypersensitive to radiation and defective in DNA repair. BRCA2 was shown to interact with RAD51 in a two-hybrid system. p53 was also shown to interact with RAD51 and inhibit RAD51's ATPase activity. However, it is not yet known how BRCA2 may be involved in p53 checkpoint and RAD51 DNA repair pathways. To facilitate the study of BRCA2 function, we generated a series of polyclonal and monoclonal antibodies specific for the BRCA2 protein. Our data indicates that the BRCA2 gene product is a 450-kDa phosphoprotein. Moreover, live cell image analysis of 293T cells transiently transfected with a GFP tagged BRCA2 full-length cDNA construct revealed that BRCA2 protein is localized to the cell nucleus.

By using a luciferase reporter assay, we show that transfection of BRCA2 into breast cancer cells expressing wild-type p53 inhibits p53 transcriptional activity, and suppresses exogenous p53 activation of p53-responsive elements. In addition, co-transfection of BRCA2 and RAD51 synergistically inhibits p53's activity. By using the antibodies generated, we demonstrated that endogenous BRCA2 can be co-immunoprecipitated in complexes containing either p53 or RAD51. These results suggest that BRCA2 functionally and physically interacts with these key components of cell cycle control and DNA repair pathways.

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The *BRCA2* gene product functionally interacts with p53 and RAD51

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ABSTRACT **Germ-line mutations in the human *BRCA2* gene confer susceptibility to breast cancer. Efforts to elucidate its function have revealed a putative transcriptional activation domain and *in vitro* interaction with the DNA repair protein RAD51. Other studies have indicated that RAD51 physically associates with the p53 tumor suppressor protein. Here we show that the *BRCA2* gene product is a 460-kDa nuclear phosphoprotein, which forms *in vivo* complexes with both p53 and RAD51. Moreover, exogenous BRCA2 expression in cancer cells inhibits p53's transcriptional activity, and RAD51 coexpression enhances BRCA2's inhibitory effects. These findings demonstrate that BRCA2 physically and functionally interacts with two key components of cell cycle control and DNA repair pathways. Thus, BRCA2 likely participates with p53 and RAD51 in maintaining genome integrity.**

Germ-line mutations in the human *BRCA1* and *BRCA2* breast cancer suppressor genes confer susceptibility to breast and ovarian cancers (1–4). Mutations in *BRCA1* and *BRCA2* are believed to be responsible for most hereditary breast cancers (3–10), which account for 5–10% of all breast cancer cases (11). About 52% of the families with four or more breast cancer cases have inherited mutations in *BRCA1*, and 32% possess *BRCA2* mutations (12). In contrast, somatic mutations in *BRCA1* and *BRCA2* are rare in sporadic cases of breast cancer (5, 13–15). Both BRCA1 and BRCA2 are up-regulated in proliferating and differentiating cells, expressed in a cell cycle-dependent manner peaking at the G1/S boundary (19–21), widely expressed during development (22–24), and essential for early embryonic development (25–29). Recent evidence indicates that BRCA1 is involved in transcriptional regulation (30, 31).

The *BRCA2* gene is composed of 27 exons and encodes a predicted 384-kDa protein possessing no obvious homology to other sequences in publicly available databases (4). There are eight repetitive units in the BRCA2 protein sequence (BRC motifs; refs. 16 and 17) and a potential nuclear localization signal within its C terminus (18). BRCA2 also possesses a putative transcriptional activation domain in exon 3, suggesting a role of BRCA2 in the regulation of gene expression (32). Evidence for a possible function of BRCA2 in DNA repair has been suggested by recent findings that Brca2 mutant mouse embryos are defective in DNA repair (27, 33, 34). Moreover, yeast two-hybrid and glutathione *S*-transferase (GST) pull-down analysis revealed that BRCA2 interacts *in vitro* with RAD51, a protein involved in DNA double-strand break repair and homologous recombination (27, 35, 36). Binding sites for RAD51 have been mapped to each of the eight BRC motifs in human BRCA2 (36) and to a C-terminal region of mouse Brca2 (27, 35).

Structural and functional characterization of the endogenous BRCA2 protein have been hampered by the large size of the protein and the lack of suitable immunological reagents for its detection. In the present studies, we generated polyclonal and monoclonal antibodies to characterize endogenous BRCA2 and identify proteins that form complexes with this protein. We demonstrate that BRCA2 is a nuclear phosphoprotein that associates *in vivo* with a significant portion of the endogenous pool of RAD51. Because an immediate cellular reponse to DNA damage is p53-mediated cell cycle arrest (37), and RAD51 has been reported to physically associate with p53 (38, 39), we also investigated whether a physical and functional relationship could be detected between BRCA2 and p53.

MATERIALS AND METHODS

Generation of BRCA2 Antibodies. Polyclonal antibodies were raised in rabbits, and mAbs were generated in mice. The peptides were conjugated to keyhole limpet hemacyanin. The three GST-fusion regions were generated by reverse transcription–PCR amplification using the following primers: 5′-GGTCAAGTTCTTTAGCTACAGGATCCACCC-3′ and 5′-CTCCATCTGGGCTCCATGTCGACCTGAAAG-3′ for GSTB1, 5′-CCTGCAACTTGTTACAGAATTCAGTC-CCC-3′ and 5′-AGGGTGAAGAGCTAGTCTCGAGTTC-CTCAA-3′ for GSTB2, and 5′-GTCAGTGAATCCACTA-GAATTCCTCCCACC-3′ and 5′-TGGTCTTGAACTCCTG-GCCTCGAGCACT-3′ for GSTB3. These regions then were subcloned into the GST-fusion protein expression vector pGEX-5X-2 or pGEX-4T-2 (Pharmacia) and verified by sequencing. All antibodies were affinity-purified.

Plasmid Constructs. pGFPB2 containing human full-length *BRCA2* cDNA fused in-frame with green fluorescent protein (GFP) was constructed as follows. A fragment at the N terminus of *BRCA2* was generated from pUCBRCA2 by PCR amplification using a high-fidelity PCR system (Boehringer Mennheim) and the following primers: 5′-GGCCAGATC-TATGCCTATTGGATCCAAAGAGAGG-3′ and 5′-GGC-CGTCGACTGCTTGTTTATCACCTGTGTCTCC-3′. This fragment was subcloned into the pEGFPC1 vector (CLONTECH) at *Bgl*II and *Sal*I sites to generate pGFPB2N. A *Sse*8387I–*Sal*I *BRCA2* fragment released from pUCBRCA2 then was ligated with pGFPB2N digested with *Bgl*II and *Sse*8387I to generate pGFPB2. Sequencing confirmed the identity of the fragment generated by PCR. A human full-length RAD51 cDNA with *Bam*HI and *Sal*I flanking was amplified from a human testis cDNA library (CLONTECH) by using *Pfu* DNA polymerase (Stratagene) and the following primers: 5′-GGCCGGATCCATGGCAATGCAGATG-CAGCTTG-3′ and 5′-GGCCGTCGACTCTTTGGCATCT-CCCACTCCAT-3′. Cloning of this cDNA fragment into pcDNA3HA generated an N-terminal hemagglutinin (HA)-

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Abbreviations: GST, glutathione *S*-transferase; GFP, green fluorescent protein; HA, hemagglutinin; RB, retinoblastoma protein.

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tagged human Rad51 construct, pHA-RAD51. A *Bgl*II–*Bam*HI fragment containing two copies of the consensus p53 binding sequence and the adenovirus major late TATA box from p50–2 (a gift from A. J. Levine, Princeton Univ., Princeton, NJ) were cloned into the pGL2 vector (Promega) to generate a luciferase reporter plasmid PGluc. GAL4luc and GAL4-VP16 were a gift from R. Davis (Univ. of Massachusetts, Worcester).

Fluorescence Microscopy. pGFPB2 was transiently transfected into 293T cells in 6-well plates containing coverslips using Lipofectamine (GIBCO). Twenty-four hours after transfection, cells were incubated with Hoechst stain (bisBenzimide Trihydrochloride Hoechst No. 33258; 5 μg/ml; Sigma) for 15 min to label nuclei. Coverslips were transferred to a Sykes-Moore perfusion chamber (Belco) mounted on the stage of a Nikon Eclipse E600 microscope and maintained at 37°C. Images were collected by using a Nikon Plan Apo ×100 oil immersion objective lens (numerical aperture = 1.4), a 1,000 × 800 pixel back-illuminated cooled charge-coupled device camera (Princeton Instruments, Trenton, NJ), and Metamorph software (Universal Imaging, Media, PA).

Luciferase Assays. Plasmid DNA was transiently transfected into cells by using Lipofectamine PLUS (GIBCO). Approximately 2×10^6 cells were cotransfected with 1 μg of reporter plasmid and 1 μg of effector plasmid except for pGFPB2 (5 μg). Cells were harvested 48 hr after transfection, and luciferase activity was measured by using a luciferase assay kit (Promega). The transfection efficiency was normalized by measuring β-galactosidase activities after the cotransfection of MFG-β-galactosidase plasmid.

Immunoprecipitation and Immunoblotting. Cell lysates were prepared in lysis buffer (50 mM Tris·HCl, pH 8.0/120 mM NaCl/0.5% Nonidet P-40/10 μg/ml aprotinin/10 μg/ml leupeptin/1 mM phenylmethylsulfonyl fluoride/1 mM sodium orthovanadate). In some cases, cells were labeled with [^{35}S]methionine (0.1 mCi/ml) or [^{32}P]phosphoric acid (0.25 mCi/ml; NEN) for 3 hr. The total protein content of the lysates was determined by bicinchoninic acid assay (Pierce). Phosphatase treatment of immunoprecipitates was performed as described (40). Cell lysates or immunoprecipitates were electrophoresed on 4%, 6%, or 10% SDS-polyacrylamide gels and transferred onto a poly(vinylidene difluoride) membrane (Millipore). ECL or ECL Plus (Amersham) was used for signal detection. Anti-GFP antibody was obtained from CLONTECH, antibodies against p53 (PAb421), DNA-dependent protein kinase, or RAD51 from Calbiochem, antibodies against the HA epitope or p53 (DO-1) from Santa Cruz Biotechnology, and anti-retinoblastoma protein (RB) antibody from PharMingen.

RESULTS

Characterization of the *BRCA2* Gene Product. To facilitate the study of BRCA2 structure and function, we generated a series of polyclonal and monoclonal antibodies by using both peptides and GST fusion proteins corresponding to various regions of the molecule (Fig. 1*A*). On Western blots of 293T cell lysates, affinity-purified polyclonal antibodies against both the N-terminal (anti-N19) and C-terminal peptides (anti-C15) recognized a band at a molecular mass much greater than that of the 220-kDa myosin standard (Fig. 1*B*). This band also was detected in MCF7 cells (see Fig. 3*C*, lane 3) and comigrated with 460-kDa DNA-dependent protein kinase (Fig. 1*C*, lane 9). However, it was not detected by anti-C15 in CAPAN-1 cells (Fig. 1*C*, lane 4), a human pancreatic tumor cell line that has lost one *BRCA2* allele and contains the 6174delT mutation in the remaining allele (41).

To confirm that this band reflected the authentic BRCA2 protein, we transfected 293T cells with the plasmid pGFPB2 containing full-length *BRCA2* cDNA fused at its N terminus to GFP or with the control vector pEGFPC1. Both anti-N19 and anti-C15 specifically recognized a band in pGFPB2-transfected, but not in vector-transfected, 293T cells at the predicted size of the GFP-BRCA2 fusion protein, approxi-

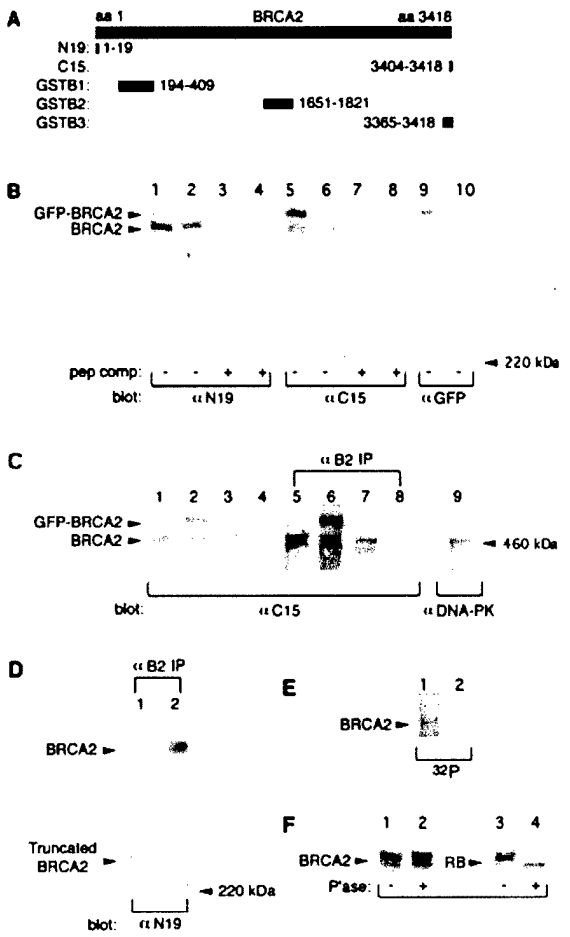

FIG. 1. Characterization of BRCA2 protein. (*A*) Diagram showing the relative positions of peptides (N19 and C15) and GST-fusion proteins (GSTB1, GSTB2, and GSTB3) used to generate antibodies against BRCA2. Among antibodies generated against GST-fusion proteins, we selected a mAb generated against GSTB2 for use in this study. (*B*) Lysates (50 μg of total protein) of 293T cells transfected with pGFPB2 (lanes 1, 3, 5, 7, and 9) or control vector pEGFPC1 (lanes 2, 4, 6, 8, and 10) were subjected to 4% SDS/PAGE, and immunoblotted with the indicated antibodies with (lanes 3, 4, 7, and 8) or without (lanes 1, 2, 5, 6, 9, and 10) homologous peptide competition. The GFP tag at the N terminus of BRCA2 may interfere with recognition of BRCA2 by anti-N19, which is specific for the N terminus of BRCA2, based on the relatively stronger signal for GFP-BRCA2 using anti-C15. (*C*) Cell lysates (50 μg of total protein; lanes 1–4), mAb B2 immunoprecipitates (from 1 mg of total protein; lanes 5–8), and DNA-dependent protein kinase immunoprecipitates (1 mg of total protein; lane 9) were resolved on the same 4% SDS/PAGE gel, and immunoblotted with anti-C15 or anti-DNA-protein kinase. Cells included 293T transfected with pEGFPC1 (lanes 1 and 5) or pGFPB2 (lanes 2 and 6), MCF7 (lanes 3, 7, and 9), and CAPAN-1 (lanes 4 and 8). (*D*) mAb B2 immunprecipitates from 2 mg of total protein of CAPAN-1 (lane 1) and MCF7 (lane 2) were resolved by 5% SDS/PAGE and immunoblotted with anti-N19. (*E*) MCF7 (lane 1) and CAPAN-1 (lane 2) cell lysates were labeled with [^{32}P]phosphoric acid, immunoprecipitated with mAb B2, resolved on a 4% SDS/PAGE, and exposed to Kodak X-Omat AR film. (*F*) BRCA2 or RB immunoprecipitates of MCF7 cells were untreated (lanes 1 and 3) or treated (lanes 2 and 4) with lambda-phosphatase, separated by 4% (BRCA2) or 8% (RB) SDS/PAGE, and immunoblotted with anti-C15 or anti-RB.

mately 27 kDa higher in mass than the native BRCA2 protein (Fig. 1*B*, lanes 1 and 5). A protein of the same size also was recognized by a polyclonal antibody against GFP (Fig. 1*B*, lane 9), confirming its identity as GFP-BRCA2. Moreover, all of these bands were specifically competed by the homologous peptides (Fig. 1*B*). Further confirmation that the proteins we had identified represent the authentic BRCA2 protein was provided by immunoprecipitation/Western blot experiments. MCF7, CAPAN-1, or transfected 293T cell lysates were immunoprecipitated with a mAb (mAb B2) raised against the GSTB2 region of BRCA2 (Fig. 1*A*), and then immunoblotted with anti-C15. The expected 460-kDa BRCA2 protein was detected in MCF7 and 293T cells but not in CAPAN-1 cells, and the 487-kDa GFP-BRCA2 fusion protein was detected only in pGFPB2-transfected 293T cells (Fig. 1*C*). All of these findings firmly established the identity of the 460-kDa protein as the product of the *BRCA2* gene.

When BRCA2 was immunoprecipitated with mAb B2 from CAPAN-1 or MCF cell lysates and immunoblotted with anti-N19, a truncated BRCA2 protein was specifically detected in CAPAN-1 cells (Fig. 1*D*, lane 1). This truncated species migrated above the 220-kDa myosin standard and had an estimated molecular mass of 230 kDa, nearly matching the predicted size of 224 kDa. These results indicate that the mutated *BRCA2* gene in CAPAN-1 cells encodes a stable truncated protein.

It should be noted that both anti-C15 and mAb B2 recognized a BRCA2 doublet in MCF7 and 293T cells (Fig. 1 *B* and *C*), suggesting the possibility of posttranslational processing, such as phosphorylation. Evidence that BRCA2 was phosphorylated derived from [^{32}P]-labeling experiments, in which a [^{32}P]-labeled band at 460 kDa was detected in autoradiographs of mAb B2 immunoprecipitates of MCF7, but not CAPAN-1 cells (Fig. 1*E*). However, when mAb B2 immunoprecipitates from MCF7 cells were treated with lambda-phosphatase, the BRCA2 doublet was not altered. Under the same conditions, the RB doublet, which is known to result from phosphorylation (42), was reduced to a single band (Fig. 1*F*). Thus, phosphorylation was unlikely to account for the BRCA2 doublet. It also should be noted that anti-N19 recognized only the upper band of the BRCA2 doublet, and that GFP-BRCA2 migrated as a single band as well (Fig. 1 *B* and *C*). Thus, the doublet likely resulted from BRCA2 amino-terminal processing or as an alternative product of the *BRCA2* gene.

Exogenously Expressed BRCA2 Localizes to the Nucleus. BRCA2 reportedly possesses a potential nuclear localization signal in its C terminus (18), and recently Bertwistle *et al.* (43) reported that BRCA2 was enriched in the nuclear fraction derived from MCF7 cells. Although our antibodies recognized GFP-BRCA2 overexpressed in transfected cells, it was not possible to reliably detect endogenous BRCA2 by standard immunofluorescent methods (unpublished data). However, the availability of the GFP-tagged *BRCA2* construct made it possible to localize the transfected GFP-BRCA2 protein in live 293T cells by fluorescence microscopy. Although GFP alone was distributed throughout the cell (data not shown), GFP-BRCA2 primarily was localized to the Hoechst-labeled nuclei (Fig. 2). These results suggest a nuclear localization of the BRCA2 protein.

***In vivo* Interaction of BRCA2 with RAD51.** BRCA2 has been reported to interact with RAD51 *in vitro* (27, 35, 36). To investigate whether these proteins form a complex *in vivo*, BRCA2 was immunoprecipitated with mAb B2 from MCF7 or CAPAN-1 cell lysates and immunoblotted with anti-RAD51. As shown in Fig. 3*A*, endogenous RAD51 was readily detected in a complex with endogenous BRCA2 in MCF7 cells (Fig. 3*A*, lane 4), whereas there was a faint signal for RAD51 coimmunoprecipitated with the truncated BRCA2 protein in CAPAN-1 cells (Fig. 3*A*, lane 3). As a specificity control, anti-IgG

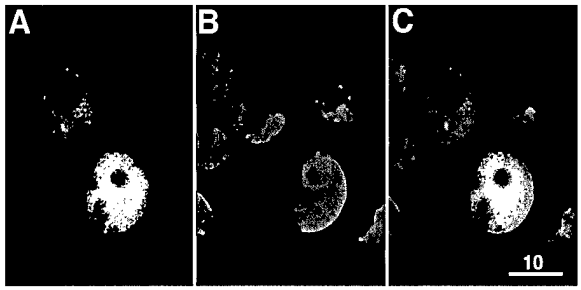

FIG. 2. Subcellular localization of exogenously expressed BRCA2. Representative images of live 293T cells transfected with pGFPB2. (*A*) Image showing the green signal of GFP-BRCA2 in transfected 293T cells. (*B*) Image showing the nuclei of cells in the same field labeled with Hoechst. Hoechst labeling is indicated as red color instead of its authentic blue color. (*C*) Overlay of the images in *A* and *B*. (Bar = 10 μm.)

failed to immunoprecipitate complexes containing RAD51 from MCF7 cells (Fig. 3*A*, lane 5). The signal intensities for RAD51 in 50 μg of cell lysates (Fig. 3*A*, lane 2) and mAb B2 immunoprecipitates from 2.5 mg of cell lysates (Fig. 3*A*, lane 4) were comparable. Thus, approximately 2% of RAD51 was present in mAb B2 immunoprecipitates. When adjusted for the approximately 10% efficiency of BRCA2 immunoprecipita-

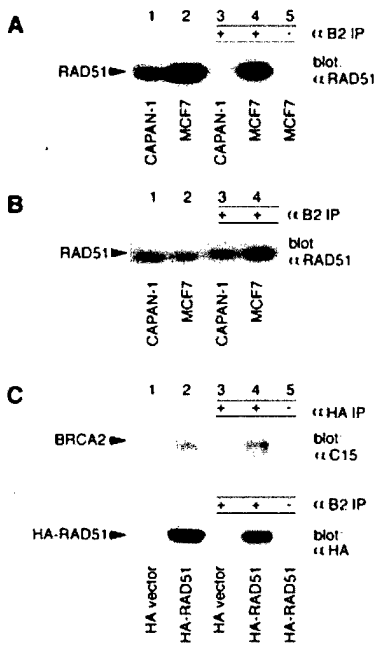

FIG. 3. *In vivo* interactions of BRCA2 with RAD51. (*A*) Cell lysates (50 μg of total protein; lanes 1 and 2) and mAb B2 (lanes 3 and 4) or anti-IgG (lane 5) immunoprecipitates (from 2.5 mg of total protein) were resolved by 10% SDS/PAGE, and immunoblotted with anti-RAD51. (*B*) Cell lysates (50 μg of total protein for CAPAN-1, and 25 μg of total protein for MCF7; lanes 1 and 2) and mAb B2 immunoprecipitates (from 4 mg of total protein for CAPAN-1, and 2 mg of total protein for MCF7; lanes 3 and 4) were resolved by 10% SDS/PAGE and immunoblotted with anti-RAD51. (*C*) 293T cells transfected with pHA-RAD51 or the control HA vector were lysed and immunoprecipitated with anti-HA, mAb B2, or anti-IgG. Lysates (lanes 1 and 2) and immunoprecipitates (lanes 3–5) were separated by 4% SDS/PAGE and immunoblotted with anti-C15, or 10% SDS/PAGE and immunoblotted with anti-HA. Anti-IgG immunoprecipitates from HA-RAD51 transfected cells were used as a specificity control (lane 5).

tion by mAb B2 (Fig. 1*C*), we estimate that at least 10–20% of RAD51 was associated with BRCA2 in MCF7 cells.

The levels of both the mutant BRCA2 protein and RAD51 were substantially lower in CAPAN-1 cells (Fig. 1*D*, lane 1; Fig. 3*A*, lane 1) than those of the corresponding proteins in MCF7 cells (Fig. 1*D*, lane 2; Fig. 3*A*, lane 2). Thus, to compare the ability of the truncated BRCA2 protein to interact RAD51 with that of the wild-type BRCA2 protein, we increased the amount of CAPAN-1 cell lysate used for coimmunoprecipitation to give similar levels of the proteins of interest to those in MCF7 cells (Fig. 3*B*, lanes 1 and 2). Under these conditions, the amount of RAD51 coimmunoprecipitated with the truncated BRCA2 in CAPAN-1 cells (Fig. 3*B*, lane 3) was comparable to that observed with wild-type BRCA2 in MCF7 cells (Fig. 3*B*, lane 4). These findings are consistent with the *in vitro* mapping of RAD51 binding sites to each of the eight BRC motifs in human BRCA2 (36) retained by the mutant BRCA2 product in CAPAN-1 cells.

We also transfected 293T cells with pHA-RAD51, an N-terminal HA epitope-tagged RAD51 construct. HA-RAD51 then was immunoprecipitated with anti-HA and immunoblotted with anti-C15. As shown in Fig. 3*C*, BRCA2 was detected in HA-RAD51 immunoprecipitates from pHA-RAD51 but not the control vector-transfected 293T cells. The presence of HA-RAD51 in BRCA2 immunoprecipitates was demonstrated in pHA-RAD51-transfected 293T cells as well (Fig. 3*C*, *Lower*). As specificity controls, none of the proteins were detected in immunoprecipitates with anti-IgG under the same conditions (Fig. 3*C*, lane 5). All of these results establish that complexes containing BRCA2 and RAD51 occur *in vivo*.

BRCA2 Forms a Complex with p53 *in vivo*. BRCA2's nuclear localization and its putative transcriptional activation domain (32) suggest that this protein may associate with a transcriptional complex. Because p53-mediated cell cycle arrest is induced after DNA damage to allow DNA repair (37), we sought to investigate whether BRCA2 and p53 might be physically present in the same complex. To do so, endogenous p53 was immunoprecipitated from MCF7 cell lysates by mAb DO-1 and immunoblotted with anti-C15. As shown in Fig. 4*A*, endogenous BRCA2 was readily detectable in p53-containing immunoprecipitates of MCF7 cells. In contrast, neither CAPAN-1 nor a p53 negative bladder cancer cell line, EJ, showed evidence of such immunocomplexes (Fig. 4*A*, lanes 4 and 6). The IgG heavy chain has a gel mobility similar to p53, making it difficult to reliably detect p53 by immunoblotting after immunoprecipitation. To perform reciprocal experiments to determine whether endogenous p53 was present in BRCA2 immunoprecipitates, we labeled cells with [^{35}S]methionine. BRCA2-containing complexes first were immunoprecipitated with anti-C15, eluted, and then immunoprecipitated with DO-1. As shown in Fig. 4*B*, a [^{35}S]-labeled 53-kDa band was observed in autoradiographs of BRCA2/p53 double immunoprecipitates of MCF7, but not CAPAN-1 or EJ cells (Fig. 4*B*, lanes 4 and 6). These findings establish that BRCA2 and p53 exist *in vivo* in the same complexes.

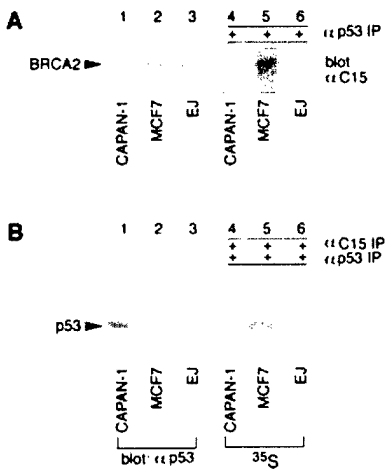

FIG. 4. BRCA2 forms a complex with p53 *in vivo*. (*A*) Cell lysates (40 μg of total protein; lanes 1–3) and p53 immunoprecipitates (4 mg of total protein; lanes 4–6) were resolved by 6% SDS/PAGE and immunoblotted with anti-C15. (*B*) Cell lysates (lanes 1–3) were separated by 10% SDS/PAGE and immunoblotted with anti-p53 PAb421. Anti-C15 immunoprecipitates from [^{35}S]-methionine-labeled cells were eluted and reprecipitated with anti-p53 DO-1. The double immunoprecipitates were resolved by 10% SDS/PAGE and subjected to fluorography (lanes 4–6).

BRCA2 Inhibits p53 Transcriptional Activity. To investigate possible functional interactions between BRCA2 and p53, we tested whether BRCA2 might act as a regulator for p53's transcriptional activity. To do so, MCF7 cells were transfected with pGFPB2 and PGluc, a luciferase reporter plasmid containing two copies of the p53-responsive element. As shown in Fig. 5, BRCA2 caused a decrease in the basal activation of p53-responsive elements by endogenous p53, when compared with transfection with the control vector pEGFPC1. BRCA2 also caused marked inhibition of exogenous p53 stimulation of PGluc. Cotransfection with BRCA2 had a similar inhibitory effect on exogenous p53-stimulated transcriptional activity in the osteosarcoma cell line, SAOS2, and the small cell lung carcinoma cell line, H1299 (data not shown). We found no difference in the level of p53 protein level expressed in cells in the presence or absence of cotransfection with BRCA2 (data not shown), excluding the possibility that inhibition by BRCA2 was the result of changes in p53 protein level. As a specificity control, GAL4luc, a luciferase reporter containing four copies of GAL4 responsive elements, was not inhibited by cotransfection of BRCA2 with GAL4-VP16, a vector expressing the GAL4 DNA binding domain fused to the transactivation domain of VP16 (data not shown). Thus, BRCA2 was not a general transcriptional inhibitor but appeared to specifically inhibit p53 transcriptional activity.

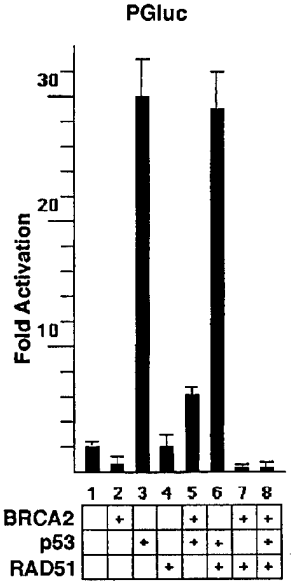

FIG. 5. RAD51 enhances BRCA2 inhibition of p53's transcriptional activity. MCF7 cells were cotransfected with the indicated plasmids and PGluc. Forty-eight hours after transfection, luciferase activity was measured as described in *Materials and Methods*.

RAD51 Enhances BRCA2 Inhibition of p53 Transcriptional Activity. If the inhibitory effect of BRCA2 on p53's transcriptional activity reflects an intersection of cell cycle checkpoint and DNA repair pathways, we reasoned that RAD51 might affect p53's transcriptional activity as well. As shown in Fig. 5, RAD51 had no effect on the activation of p53-responsive elements by endogenous p53 in MCF7 cells or in response to exogenous p53. However, cotransfection of RAD51 with BRCA2 caused nearly complete inhibition of PGluc activation by either endogenous or exogenous p53. These results indicate that RAD51 enhances BRCA2 inhibition of p53's transcriptional activity.

DISCUSSION

Abnormalities caused by targeted disruption of the *Brca2* gene include increased sensitivity to DNA damage induced by ionizing irradiation, UV light, and other genotoxic agents (27, 33, 34). The accumulation of double-strand DNA breaks and chromosomal abnormalities combined with the lack of obvious checkpoint or apoptotic response abnormalities in *Brca2* mutant cells have implied a role of BRCA2 in DNA repair (33, 34). Recent findings that BRCA2 and RAD51 interact *in vitro* have suggested further that BRCA2 may be involved in RAD51-mediated repair pathways (27, 35, 36). In this study, we identified the *BRCA2* gene product as a 460-kDa nuclear phosphoprotein that forms a complex with RAD51 *in vivo*. While this manuscript was in preparation, Chen *et al.* (44) reported detection of BRCA2 as a nuclear protein, consistent with our findings. They also reported detection of immunocomplexes containing BRCA2 and RAD51 (44). Our findings established that a major fraction of endogenous RAD51 is associated with the endogenous BRCA2 protein, implying that this interaction is functionally significant.

We demonstrated further that CAPAN-1 tumor cells, which contain a *BRCA2* mutation producing a truncated product, express a single BRCA2 immunoreactive species of around 230 kDa, consistent with the predicted size of the mutant. Of note, the mutant protein, though expressed at low level, interacted with RAD51 as efficiently as the wild-type BRCA2. This mutant would be predicted to retain all eight repetitive BRC motifs that have been mapped *in vitro* to be RAD51 binding sites in human BRCA2 (36). These results point to a possible mechanism by which truncated BRCA2 mutants found commonly in tumors (Breast Cancer Information Core) actually may sequester RAD51 in a nonfunctional complex.

The p53 tumor suppressor gene is known to mediate cell cycle arrest after DNA damage (37), and there are reports that p53 can be detected in complexes with RAD51 (38, 39). We established here that p53 exists in *in vivo* complexes containing BRCA2. Moreover, a functional interaction between BRCA2 and p53 was demonstrated by evidence that BRCA2 inhibits p53 transcriptional activity. These results are consistent with the possibility that BRCA2 may act to limit the length or severity of p53-mediated cell cycle arrest after DNA damage. Other studies have shown that *Brca2* mutant mouse embryos exhibit a growth arrest phenotype (27–29, 33, 34), and that this phenotype is less severe in *Brca2/p53* double mutant mouse embryos (28). In addition, *Brca2* mutant embryo cells exhibit increased $p21^{WAF1/CIP1}$ levels associated with a defect in cell proliferation (29, 33, 34). All of these findings are consistent with a role of BRCA2 in down-regulating p53 transcriptional activity.

BRCA2, *RAD51*, and *BRCA1* have similar patterns of expression (45), and mouse mutants of each of these genes exhibit embryonic lethality (25–29, 46, 47) that can be partially rescued in a *p53* null background (28, 47, 48). These findings imply that all of these molecules have related functions. BRCA1 and BRCA2 have been reported to have transcriptional activity potential (30–32). Although RAD51 has been shown to be a component of the RNA polymerase II holoenzyme (49), it is not itself known to have transcriptional activity (45). We did not detect any effect of RAD51 on p53 transcriptional activity. However, we did observe cooperative down-regulation of p53's activity by RAD51 and BRCA2, providing evidence that RAD51 is coupled to transcription pathways through its interactions with BRCA2 and p53. Thus, BRCA2 appears to serve as a regulator linking both cell cycle control and DNA repair pathways.

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